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High-dose Nitric Oxide Inhalation Increases Lung Injury after Gastric Aspiration

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Background: **Inhaled nitric oxide is often used in patients with adult respiratory distress syndrome. However, nitric oxide also may be significantly toxic, especially if administered concurrently with hyperoxia. The authors evaluated the isolated effect of nitric oxide and the combined effects of nitric oxide and hyperoxia on lung injury in rats after acid aspiration.**

Methods: **Animals were injured by instillation of 1.2 ml/kg hydrogen chloride in low-*pH* saline (the acid group) or acidified gastric particles (the casp group) into the lungs under halothane anesthesia *via* a tracheal catheter. Controls received no injury vehicle but rather underwent the surgical process. After recovery from anesthesia, the animals were exposed to 20% or 90% oxygen with or without 20, 40, or 80 ppm nitric oxide for 5 h. The permeability index, alveolar–arterial oxygen difference, the ratio of oxygen pressure to the inspired fraction of oxygen, and the ratio of wet to dry weight were assessed 5 h after injury as indices of lung injury. Data were assessed using analysis of variance.**

Results: **Each group included 6–10 rats. Exposure to nitric oxide (80 ppm) in air increased protein permeability in the lungs to a permeability index of 1.42 ± 0.12 after acid aspiration. The combination of nitric oxide (80 ppm) and hyperoxia further increased protein leakage to a permeability index of 2.1 ± 0.25. Exposure to lower concentrations of nitric oxide (*e.g.*, 20 and 40 ppm) increased the permeability index of the lungs (1.44 ± 0.21, 1.75 ± 0.29, respectively) in the presence of hyperoxia, although it did not affect the permeability index of the lungs during exposure to air. Pretreatment of animals with deferoxamine and methylene blue partially inhibited the adverse effect of hyperoxia and nitric oxide, which suggested a complex underlying mechanism involving both reactive-species generation and pulmonary vasomotor changes.**

Conclusions: **These results show that inhaled nitric oxide at 80 ppm for a short duration (5 h) increases the severity of the inflammatory microvascular lung injury after acid aspiration. The pulmonary damage is exacerbated further in the presence of high oxygen concentrations. Although lower concentrations of nitric oxide did not increase the extent of lung injury, longer exposure times need to be assessed. (Key words: Acid; hyperoxia; inflammation; neutrophil; rats.)**

ADULT respiratory distress syndrome develops in approximately one third of patients who aspirate gastric contents during the perioperative period.[1–3] The mainstay of current treatment in such patients includes mechanical ventilation, high-inspired concentrations of oxygen, and positive end-expiratory pressure. Despite therapy, 30–70% of these patients die as a result of respiratory failure.[4]

Nitric oxide (NO) inhalation has been used widely to treat primary pulmonary hypertension and is being evaluated for the treatment of adult respiratory distress syndrome in the critical care setting.[5,6] However, the beneficial effect of this vasoactive compound in adult respiratory distress syndrome is controversial.[7] Nitric oxide is a reactive compound, therapeutically administered as a gas, and is produced endogenously from L-arginine by NO synthase. Although NO is critical to host defense, this compound may become autotoxic in certain genetic and acquired diseases.[8]

A rat model of gastric aspiration has been established and characterized extensively in our laboratory.[9,10] The role of acute inflammation in the pathogenesis of aspiration pneumonitis has been assessed previously.[9,11] In addition, we have shown that exposure to high ambient concentrations of oxygen for 2 h after aspiration of

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acidic gastric contents significantly increases the extent of lung injury in the awake, nonmechanically ventilated animal.[12]

The goal of the current study was to evaluate the effect of inhaled NO in acute lung injury after aspiration of gastric contents. We hypothesized that exposure to NO would modify the severity of injury by its interaction with reactive species of oxygen in the presence of hyperoxia. In addition, the contribution of inhaled NO to lung injury in the presence of increased oxidant stress has been tested by simultaneous exposure of the rats to oxygen and NO after acute lung injury.

Materials and Methods

The experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo based on National Institutes of Health guidelines. Pathogen-free male Long-Evans rats (weight, 250–300 g) were anesthetized with halothane, and lung injury was induced by intratracheal injection of 1.2 ml/kg hydrogen chloride in normal saline, *p*H = 1.25 (the acid group), or 40 mg/ml combined acidified (*p*H = 1.25) small (approximately 10 μm) gastric particles (the casp group) through a 22-gauge needle at a 60-degree upright position.[13] Sham injury was used for control animals and included tracheotomy without instillation of any injury vehicle. Halothane exposure lasted no longer than 15 min. To measure pulmonary protein permeability, rats were injected with 0.05 μCi ^{125}I-albumin mixed with 2% bovine serum albumin *via* the dorsal penile vein using a 26-gauge needle. The groups of rats that required a specific inhibitor received the proposed treatment at the time of injury *via* the same route.

The animals were allowed to recover from anesthesia and were exposed to 0, 20, 40, and 80 ppm NO combined with air or 90% oxygen for 5 h after intrapulmonary deposition of the injury vehicle in a controlled atmosphere chamber. Exposure chambers measured approximately 1 cubic foot in volume and were continuously vented with a fresh gas flow of 5 l/min. Temperature was maintained at 25°C by a thermoregulator-controlled heating lamp. The concentration of NO and nitrous dioxide (NO_2) were monitored continuously using a chemiluminescence NO, NO_2, and nitrogen oxide analyzer (Thermo Environmental, Franklin, MA) in the chambers. The NO_2 levels were less than 5 ppb in all experimental settings.

At the end of 5 h, rats were exposed to 85% oxygen for 15 min before arterial blood sampling from the descending aorta using an 18-gauge needle. Arterial blood gas was analyzed and alveolar oxygen tension was calculated. The ratio of the partial pressure of oxygen in arterial blood to the inspired oxygen fraction and the alveolar–arterial oxygen difference, $D(A-a)O_2$, were used as indicators of intrapulmonary shunting. The extent of lung injury was further determined by assessment of pulmonary alveolar leak of radiolabeled protein from the intravascular space and the ratio of wet to dry lung weights. Protein leak through the alveolar–capillary membrane was measured as the ratio of radioactivity (in counts per minute) in the harvested and saline-perfused lungs to the radioactivity of 1 ml blood from the same animal, defined as the protein permeability index (PI). Previous data in lung injury models showed a close correlation among the degree of change in PI, the extent of the inflammatory responses, and tissue damage, as assessed by histologic criteria.[14,15]

High-pressure Liquid Chromatography Assay for Protein Nitrosylation

In a separate set of experiments, saline-perfused lungs of control and experimental animals (described before) were excised and frozen by immersion in liquid nitrogen. Another group was added in which the lungs were excised and instilled with 0.3 ml morpholinosydnoimine (1 mM) and −HCl (0.005 mM, *p*H = 2.25; SIN-1, Calbiochem-Novabiochem, La Jolla, CA), incubated at room temperature for 1 h, and frozen in liquid nitrogen. SIN-1 is a compound that decomposes on neutralization into NO and superoxide radicals, which react to produce peroxynitrite.[16] These samples were used as positive controls for *in situ* nitrosylation of tyrosine residues by peroxynitrite. Whole lungs were homogenized, on ice, in 5 ml phosphate buffered saline, *p*H = 7.4 with 6 × 10 s pulses (the highest setting) of a tissue homogenizer (Polytron, PT-2000; Brinkman Instruments, Westbury, NY). The tissue homogenates were centrifuged at 2000*g* for 15 min at 4°C and the supernatant was collected. The total protein concentration was measured in the supernatant using a Pierce (Rockford, IL) protein assay protocol, and 5 μg soluble lung protein was subjected to vapor-phase acid hydrolysis for 20 h in 115°C, with a crystal of phenol present in the reaction vial. Samples and nitrotyrosine standards were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and separated by high-pressure liquid chromatography. The 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate re-

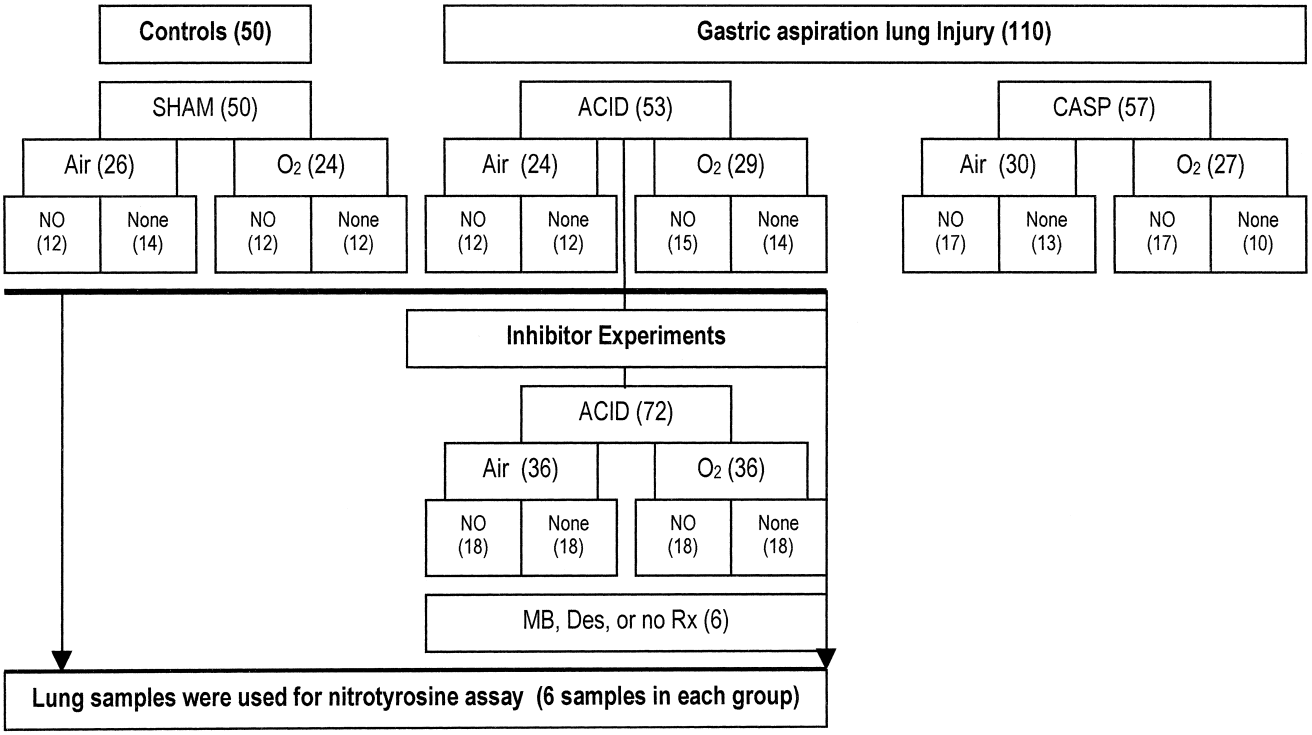

Fig. 1. The design of the study and experimental groups. The number of animals in each group is shown in parentheses.

agent and mobile phase buffer were part of an AccQ-Fluor Reagent Kit (Waters Chromatography Division, Milford, MA), and the column was a 3.9 × 150 mm, 4 μm Nova-Pak C18 AccQ · -Tag column (Millipore Corp., Waters Chromatography Division). The Peak retention time was 6.80 ± 0.03 min for tyrosine and 8.85 ± 0.02 min for nitrotyrosine. The nitrotyrosine concentration was used as an index of protein nitrosylation and was determined by interpolation of the nitrotyrosine-integrated peak area of the sample with the standard curve.

Inhibitor Experiments

To gain a better understanding of the role that NO may play in the development of the lung injury we were evaluating, we used specific inhibitors (all obtained from Sigma Chemical Co., St. Louis MO). Nω-nitro L-arginine methyl ester, an inhibitor of nitric oxide synthase, was injected intravenously (300 mg/kg) with the radioactive bovine serum albumin just before injury to prevent endogenous production of NO. To block peroxynitrite production, 30 mg/kg deferoxamine was administered in the same manner. This dose was shown previously to provide maximum protection against hyperoxia-induced increase in lung injury after acid aspiration.[12] Because most of direct vasoactive effects of NO are mediated through activation of soluble guanyl cyclase, we used intravenous methylene blue, a known inhibitor of cyclic guanosine monophosphate formation, to evaluate the role of direct effects of NO on acid-induced lung injury.[17,18] Methylene blue also was injected with the tracer. Because of the relatively short half-life of this compound (45 min), the dose was repeated 2 h after injury. The extent of lung injury was assessed 5 h after instillation of the aspirate in all inhibitor experiments.

Statistical Analyses

Ten to 15 rats were studied in each exposure group and six rats were examined in the inhibitor experiments and nitrotyrosine group (fig. 1). The number of the rats in each group was selected based on a power analysis accepting a 20% β error and 5% α error. Data were expressed as the mean ± SD and analyzed by factorial analysis of variance. The Bonferroni–Dunn test was used for *post hoc* analysis of the injury and the treatment. An f test was performed and variance was calculated. Multiple Student *t* tests were used to compare any specific groups to their controls assuming unequal variance. Simple regression analysis was performed to evaluate the

Table 1. Arterial Blood Gas Analysis of the Injury Groups Exposed to Air for 5 h following Injury

Groups of Injury	N	*p*H	Pa_{CO_2} (mmHg)	Pa_{O_2}/FI_{O_2} (mmHg)	$D(A\text{-}a)_{O_2}$ (mmHg)
Sham	12	7.32 ± 0.01	52.3 ± 2.0	395 ± 41	193 ± 33
Acid	11	7.31 ± 0.02	49.4 ± 1.9	285 ± 34	279 ± 28*
Casp	13	7.30 ± 0.01	60.2 ± 2.4	113 ± 18	408 ± 15*

All the animals were exposed to 85% oxygen with a nose cone prior to arterial blood sampling. Barometric pressure was measured daily in the laboratory.
* Significant difference ($P < 0.05$).

dose dependency of NO during hyperoxia. The Fisher exact test was used to analyze mortality data. Null hypotheses were rejected at $P < 0.05$.

Results

Intrapulmonary deposition of acid or acidified gastric particles produced significant inflammatory lung injury as assessed by PI and the lung wet–dry weight ratio. Oxygenation was also affected and pulmonary shunting increased by acidic injuries. Aspiration of acid or acidified gastric particles resulted in a detectable decrease in the ratio of the partial pressure of oxygen in arterial blood to the inspired oxygen fraction and a marked increase in $D(A-a)O_2$ compared with sham-treated rats (table 1). Overall, the mortality rate varied between 5% and 10% in all injury groups exposed to air. Four of 14 (28%) rats injured with acidified gastric particles and exposed to NO in 90% ambient oxygen died within the 5-h monitoring time, which is significantly higher than in the other groups ($\chi^2 = 4.97$; $P < 0.05$).

The Effect of Hyperoxia Exposure in Lung Injury

Untreated, acid-injured rats exposed to 90% oxygen had increased PI values and wet–dry weight ratios compared with similarly injured rats exposed to air (0.89 ± 0.15 and 5.69 ± 0.15 compared with 1.33 ± 0.43 and 5.95 ± 0.14, respectively). After intrapulmonary deposition of the acidified gastric particles, a 5-h exposure to 90% ambient oxygen produced a significant increase in protein leakage (3.44 ± 0.28 *vs.* 4.66 ± 0.37; $P < 0.05$), whereas the lung wet–dry weight ratios did not change significantly compared with air-exposed rats. The partial pressure of carbon dioxide in arterial blood was also elevated (71.4 ± 2.7 *vs.* 49.4 ± 1.9 mmHg) in response to higher inspired oxygen fraction exposure levels, which resulted in a relative respiratory acidosis (*p*H 7.26 ± 0.03 *vs.* 7.31 ± 0.04). A similar effect was also observed with other types of lung injury (table 2).

The Effect of Nitric Oxide Exposure on Lung Injury

The NO_2 levels in the exposure chambers were less than 5 ppb in all experimental groups exposed to nitric oxide. Five-hour exposure to NO (80 ppm) delivered in air increased the PI from 0.89 ± 0.15 to 1.44 ± 0.49 ($P < 0.05$) and increased the wet–dry weight ratio from 5.69 ± 0.05 to 6.17 ± 0.50 ($P < 0.05$) after intratracheal instillation of hydrogen chloride compared with similarly injured, air-exposed rats (fig. 2). Rats that received acidified gastric particles and exposed to NO in air for

Table 2. Arterial Blood Gas Analysis of the ACID- and CASP-injured Rats Exposed to NO in Air or Oxygen for 5 h

Exposure	N	*p*H	Pa_{CO_2} (mmHg)	Pa_{O_2}/FI_{O_2} (mmHg)	$D(A\text{-}a)_{O_2}$ (mmHg)
Acid					
Air	11	7.31 ± 0.02	49.4 ± 1.9	285 ± 34	279 ± 28
NO in air	11	7.28 ± 0.03	49.8 ± 1.8	274 ± 36	285 ± 28
100% oxygen	14	7.26 ± 0.01*	71.5 ± 2.7*	241 ± 19	289 ± 14
NO in 100% oxygen	15	7.26 ± 0.02*	71.8 ± 2.4*	225 ± 26*	301 ± 20
Casp					
Air	13	7.30 ± 0.01	60.2 ± 2.4	113 ± 18	408 ± 15
NO in air	13	7.30 ± 0.01	61.3 ± 2.5	85 ± 9	429 ± 8
100% oxygen	10	7.28 ± 0.01*	69.8 ± 2.2*	73 ± 6	429 ± 4
NO in 100% oxygen	12	7.20 ± 0.04*	82.6 ± 6.4*	59 ± 8*	427 ± 7

All the animals were exposed to 85% oxygen with a nose cone prior to arterial blood sampling. Barometric pressure was measured daily in the laboratory.
* Significant difference ($P < 0.05$).

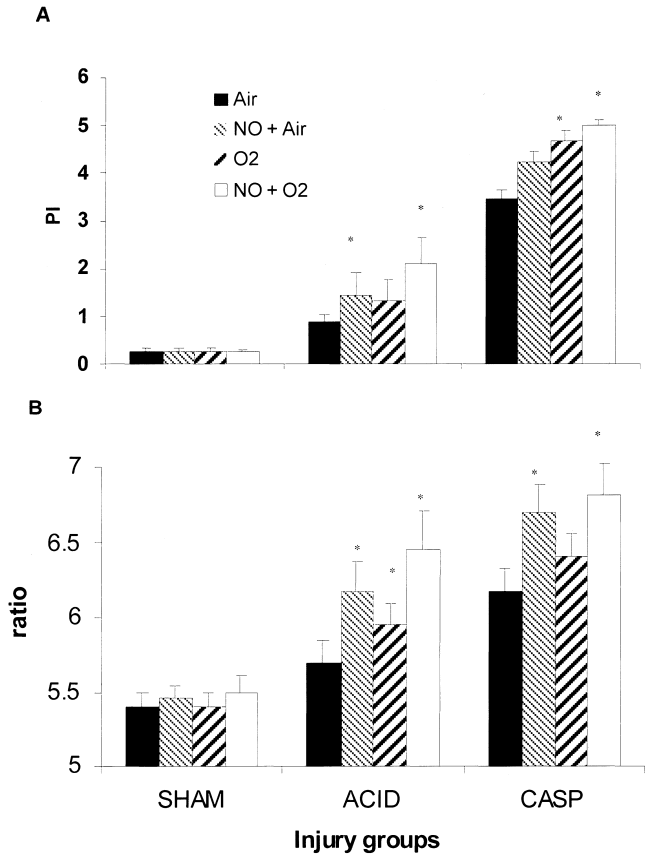

Fig. 2. Rats (n = 11–15) were injured with hydrogen chloride (the acid group) or acidified gastric particles (the casp group) and then exposed to air, nitric oxide (80 ppm) in air, 90% oxygen, or (80 ppm nitric oxide) in 90% oxygen for 5 h. Lung injury was assessed according to (*A*) pulmonary microvascular protein leakage (permeability index) and (*B*) the lung wet–dry weight ratio. Data are presented as the mean ± SD, and statistical significance is shown by asterisks.

5 h did not show an increase in PI but rather a moderate increase in the wet–dry weight ratio (from 6.17 ± 0.10 to 6.70 ± 0.15; $P < 0.05$) compared with air-exposed controls (fig. 2).

Because the acid group showed a maximum effect to NO exposure, the dose response was examined only in rats injured with hydrogen chloride. A dose-dependent effect of NO exposure could not be demonstrated without increased ambient oxygen concentrations. Exposure of acid-injured rats to 20 or 40 ppm in air for 5 h resulted in no increase in the PI (0.88 ± 0.11 and 0.92 ± 0.14, respectively) or the wet–dry weight ratio (5.62 ± 0.09 and 5.70 ± 0.12) when compared with air-exposed controls. However, a trend of decline in oxygenation was observed with both concentrations of NO after acid injury that was not statistically significant.

The Effect of Concurrent Exposure to Hyperoxia and Nitric Oxide in Lung Injury

Lung damage was maximal in every injury group tested when the animals were exposed to an ambient mixture of 80 ppm NO and 90% oxygen for 5 h. The PI increased from 0.89 ± 0.15 to 2.10 ± 0.55 in acid-injured animals ($P < 0.01$), and from 3.44 ± 0.28 to 4.99 ± 0.41 after injury with the acidified gastric particles ($P < 0.05$) (fig. 2). Similarly, the wet–dry weight ratio increased from 5.69 ± 0.15 to 6.45 ± 0.26 in acid-injured and from 6.17 ± 0.13 to 6.82 ± 0.11 in the acidified gastric particles model ($P < 0.05$). We were able to show a dose response to NO when it was combined with increased ambient oxygen ($R^2 = 0.88$, $P < 0.01$; fig. 3). Exposure to lower concentrations of NO (20 ppm) in hyperoxia slightly increased the PI (1.44 ± 0.21 *vs.* 1.33 ± 0.41; P = NS). However, higher concentrations of NO (40 and 80 ppm) produced significant increase in PI (1.75 ± 0.29 and 2.10 ± 0.55, respectively; $P < 0.05$) compared with hyperoxia-exposed controls after acid injury. Lung wet–dry weight ratios were not different from those in the acid-injured and hyperoxia-exposed controls.

Nitric oxide exposure for 5 h in conjunction with either 90% oxygen or air resulted in a significant increase in the alveolar–arterial oxygen difference after acid and acidified gastric particle injury, compared with their controls without NO exposure ($P < 0.05$) (table 2). Furthermore, the oxygenation ratio (partial pressure of oxygen in arterial blood to inspired oxygen fraction) decreased significantly after 5 h of exposure to NO in conjunction

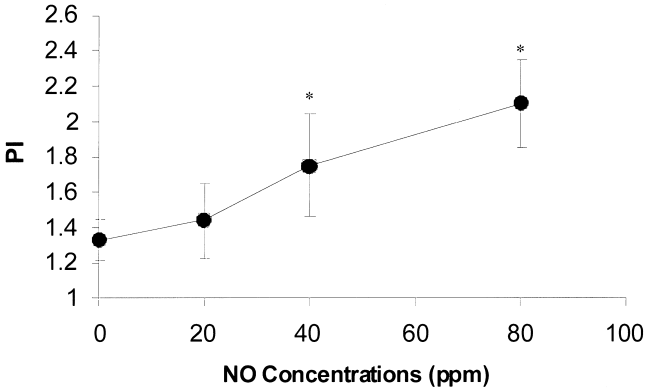

Fig. 3. Six rats were injured with hydrogen chloride and exposed to 20 and 40 ppm nitric oxide (NO) in 90% oxygen. Twelve rats were evaluated in either 0 or 80 ppm nitric oxide with 100% oxygen groups. Exposure time was 5 h in all groups. Lung injury was assessed by the permeability index (PI). Significant differences compared with air-exposed controls are indicated by asterisks ($P < 0.05$). Simple regression analysis of the permeability index showed a significant dose response to nitric oxide when it is associated with hyperoxia ($R^2 = 0.88$, $P < 0.01$).

with 90% oxygen in all injury models compared with their air-exposed and injured controls without NO exposure ($P < 0.05$). There were mild increases in $D(A-a)O_2$ associated with exposure to lower concentrations of NO in the hyperoxia group (data not shown).

Protein Nitrosylation Associated with Exposure to Hyperoxia and Nitric Oxide

In this set of experiments, six rats were used in each experimental group. Nitrotyrosine concentrations were increased in lungs injured with instillation of hydrogen chloride and exposed to NO (80 ppm) and 90% oxygen (323.5 ± 169.1 nmol/mg protein) when compared with the lungs with a similar injury and exposure to air or oxygen without NO (57.2 ± 8.9 and 88.9 ± 16.7 nmol/mg protein; $P < 0.05$). Nitrotyrosine levels in the acid-injured lungs exposed to NO and 90% oxygen were similar to the levels in the lungs treated with an endogenous donor of peroxynitrite, SIN-1 (fig. 4).

Inhibition Experiments

The changes in the protein PI and wet–dry weight ratio were the most significant in the presence of low-*p*H solutions. Therefore, rats with acid injury were selected to be treated with specific inhibitors to evaluate the role of NO in the generation of reactive species and in alteration of pulmonary hemodynamic status. In these experiments, the PI was used as the main index of inflammation because of its high sensitivity. Wet–dry weight ratios of the lungs are also reported, but the sensitivity of this parameter has been very low. Oxygenation data was not obtained for these rats because of a radioactivity contamination hazard. Treatment of animals with 300 mg/kg Nω-nitro L-arginine methyl ester before acid injury and the 5-h exposure to 90% oxygen resulted in a 21% reduction in PI (1.33 ± 0.11 to 1.05 ± 0.1; $P < 0.05$). This suggested a role for endogenous NO in the mediation of hyperoxia–acid-induced injury. In rats exposed to NO for 5 h after pulmonary deposition of acidic solutions, administration of deferoxamine decreased the protein leakage and water content of the lungs to the same extent with or without coexposure to hyperoxia. In air-exposed animals that received NO, deferoxamine pretreatment reduced the protein leakage from 1.44 ± 0.49 to 1.15 ± 0.25 ($P < 0.05$) without any improvement in lung wet–dry weight ratio (6.17 ± 0.20 *vs.* 5.98 ± 0.13). Deferoxamine decreased the protein leakage to levels comparable to an air-exposed animal after exposure of the rats to hyperoxia for 5 h after intratracheal instillation of acid when NO was not added to the inspiratory gas mixture. However, when acid-injured rats were exposed to NO and high concentrations of inspiratory oxygen simultaneously, deferoxamine only partially reversed the additional lung injury associated with breathing these gases (fig. 5).

Methylene blue was used to evaluate the role of soluble guanyl cyclase activation and its role in the observed alterations in protein permeability, wet–dry weight ratios, and increased pulmonary shunting. Methylene blue treatment produced a partial inhibition of lung injury associated with administration of NO to acid-injured animals (fig. 5). Interestingly, in animals exposed to hyperoxia in the absence of NO after acidic injury, administration of methylene blue caused significant increases in protein permeability and the wet–dry weight ratio of the lungs.

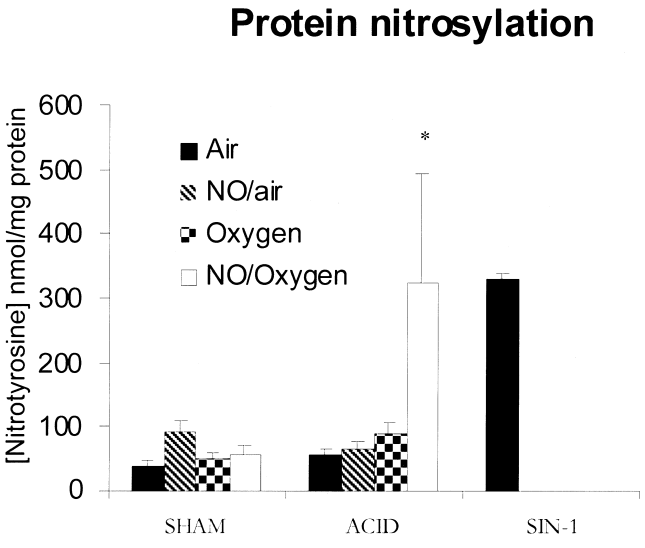

Fig. 4. The nitrotyrosine concentration was determined in whole lung homogenates of 96 rats as an index of protein nitrosylation and peroxynitrite generation. Eight rats were examined in each group. Six pairs of lung were excised and exposed to a nitric oxide donor (SIN-1) *ex vivo* for 1 h and then homogenized and used as internal positive controls. Exposure to 80 ppm nitric oxide in 90% oxygen results in significant increases in the nitrotyrosine concentrations that are similar to those in SIN-1–positive controls. Data are expressed as the mean ± SD, and asterisks indicate significant differences.

Discussion

Nitric oxide therapy has been explored as a treatment modality for patients with adult respiratory distress syndrome.[19,20] Several investigators have questioned the efficacy of such treatment for this condition.[21,22] We have shown that inclusion of NO (80 ppm) in respiratory

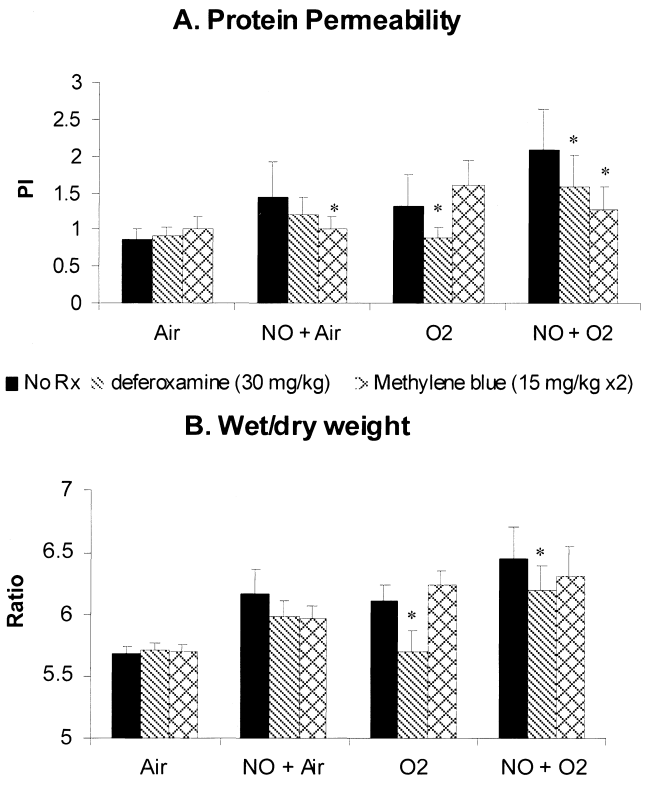

Fig. 5. Six to eight rats were injured with intrapulmonary instillation of hydrogen chloride and were treated with 30 mg/kg deferoxamine or 15 mg/kg methylene blue at the time of the injury. They were then exposed to air, 80 ppm nitric oxide (NO) in air, 90% oxygen, or 80 ppm nitric oxide in 90% oxygen for 5 h. The extent of injury was evaluated (*A*) by the permeability index (PI) and (*B*) by the lung wet–dry weight ratio. Data are expressed as the mean ± SD, and asterisks indicate significant differences.

gases increases the extent of lung injury after instillation of acidic solutions. The injurious effect of high-dose NO is more prominent in the presence of high ambient concentrations of oxygen. Although lower doses of NO (20 and 40 ppm) in air do not increase pulmonary protein leakage and lung wet–dry weight ratios, when administered with 90% oxygen, lower concentrations of NO appear to enhance lung injury in a dose-dependent manner.

We have shown that hyperoxic exposure results in an increase in the extent of lung injury after pulmonary acid injury.[12] Nω-nitro L-arginine methyl ester abolishes this injury, signifying a role for endogenous NO. In this study, Nω-nitro L-arginine methyl ester decreased protein leakage secondary to a 5-h exposure to hyperoxia after an acid injury. The beneficial effects of Nω-nitro L-arginine methyl ester have not been shown with longer hyperoxia exposure.[23] Nozik *et al.*[24] found that L-arginine increases injury in the isolated rabbit lung during hyperoxia. However, these results indicate that endogenous NO plays a role in hyperoxia-mediated injury.

The interaction between exogenous NO and hyperoxia is controversial. McElroy *et al.*[25] showed that administration of NO decreased protein leakage in an oxygen toxicity model in rats. Exposure to high-dose NO (100 ppm) increased the survival rate of rats after a 120-h exposure to hyperoxia from 8% to 70%.[26] Such protection was not observed in rats exposed to lower dose of NO (10 ppm). The presence of acute inflammation is the fundamental difference between this model and the rat model used in the current study. We postulate that the toxic effects of NO and oxygen are enhanced in the presence of an ongoing inflammatory process, an abundance of toxic mediators, and high concentrations of molecular oxygen.

Interspecies differences in responsiveness to NO should be considered when applying experimental findings from rats (which are more NO responsive) to humans (who are less NO responsive).[27,28] However, there is evidence that human tissues are also subject to NO- and hyperoxia-related toxicity. A synergistic cytotoxicity is described with NO and hyperoxia on cultured human alveolar epithelial cells that is associated with increases in nitrosylated proteins.[29] These cells die prematurely (2 days) when exposed to a combination of NO and 95% oxygen compared with a normal 6-day survival time in air-exposed controls. In another study, isolated human neutrophils exposed to NO and hyperoxia for 24 h also underwent apoptotic changes.[30] Doses less than 5 ppm did not increase the rate of apoptosis in these cells. Treatment with recombinant superoxide dismutase decreased the rate of apoptosis, indicating a role for peroxynitrite generation as the underlying mechanism. The use of superoxide dismutase in other NO–hyperoxia models has been shown to be protective.[31]

Nitric oxide is a potent electron donor, and in the presence of oxygen forms nitrous dioxide, which is also toxic. Even low concentrations of NO_2 (0.2 to 2 ppm) can produce lung injury by irritating the airway epithelium and altering the function of surfactant.[32,33] The rate of NO_2 formation is directly proportional to the oxygen and NO concentrations.[34] We carefully monitored the NO_2 concentration in the exposure chamber to avoid exposing the experimental animals to this compound; however, the local generation of this metabolite is possible and may contribute to the lung injury.

In addition, NO can react with the superoxide ion to form peroxynitrite, a reactive species that also causes

injury to various components of alveolar epithelium and alters pulmonary surfactant function.[22,33] Administration of high concentrations of NO (80 ppm) promotes the reaction between superoxide and NO.[35–37] We present evidence for the generation of peroxynitrite associated with exposure of injured lungs to NO and hyperoxia. Nitrotyrosine has been used widely as a marker of peroxynitrite activity in tissues.[38,39] Our findings support the hypothesis that in animals exposed to NO and hyperoxia after acid injury, protein nitrosylation increases to a significant level that is comparable to SIN-1–treated positive controls. This finding clearly indicates that peroxynitrite generation is critical in this injury and further potentiated by exogenous exposure to NO and hyperoxia.

Alteration of pulmonary perfusion by increasing the flow to the injured areas of lungs may also contribute to pulmonary protein leakage and edema. The hemodynamic effects of NO are directly mediated at lower concentrations of this reactant.[40] Because the lung injury is more evident in high doses of NO, we predict that the indirect effects of NO (*e.g.*, generation of peroxynitrite, or NO_2) play an important role in protein leakage and tissue edema. To examine this hypothesis further, deferoxamine and methylene blue were used to inhibit the oxidative and hemodynamic effects of NO, respectively.[41,42]

Deferoxamine alters the electron chain necessary for the generation of peroxynitrite from NO and reactive oxygen intermediates by limiting iron availability. Administration of deferoxamine partially decreases the extent of lung injury after exposure to NO and hyperoxia. Methylene blue administration to the rats exposed to NO alone or in combination with oxygen decreased the extent of lung injury induced by acidic solutions and gastric particulate material. This finding suggests that changes in pulmonary vasomotor responses partially contribute to the NO-mediated increase in the extent of lung injury. Interestingly, the protein leakage and wet–dry weight ratio of the lungs treated with methylene blue increase when the animals are exposed to hyperoxia alone after acidic injury. This may be the result of inhibition of ascorbic acid, glutathione, and other native antioxidants by this compound.[43,44]

We conclude that exposure to NO does not offer any benefits in the term of protein permeability, lung water content, and intrapulmonary shunting, especially when NO is administered in conjunction with high concentrations of oxygen, a scenario that is often found in the clinical setting. In this study, the exposure to NO was for a short period (5 h). We speculate that longer duration of exposure to NO after an acute inflammatory lung injury may be more deleterious and result in profound damage to pulmonary epithelium, as is seen with hyperoxia. Of concern is the finding that NO at high concentrations (80 ppm) may also exacerbate the inflammatory response and increase the lung damage after aspiration pneumonitis. This adverse effect seems to be due to a combination of the effects of NO on the generation of reactive species of oxygen and nitrogen and on the pulmonary vasculature.

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